THE RESPONSE OF THE HUMAN EPIDERMIS TO THE APPLICATION OF CARCINOGENIC HYDROCARBONS

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Hydrocarbons that have a high carcinogenic index in the mouse and rat have been studied extensively (6). Their action in man is largely that of conjecture for very few factual data are available. Certain strains of mice are exceptionally prone to develop carcinomatous growths after the application of various hydrocarbons. Rabbits, in contrast, are quite resistant.

3-methylcholanthrene, one of the most potent of all carcinogenic hydrocarbons was first synthesized by Wieland and Dane (14) from desoxycholic acid, a normal constituent of human bile. This same carcinogenic hydrocarbon has been chemically synthesized from cholic acid and derivatives of cholesterol (14). The possibility (14) that sterols may be degraded through a process of abnormal metabolism to a substance capable of initiating cancer has been entertained by many investigators.

Malignant growths in mice have resulted from local application and the injection of a large number (6, 43, 41, 39) of unrelated substances. Estrogens (25, 26) and certain petroleum products (48, 43) regularly produce tumors in these animals. At the present time the macromolecular compounds have come under study (9, 10). These compounds such as cellophane (9), dacron (9) and polyethyelene and the plasma extenders (dextran and polyvinyl pyrrolidones) (10), produce carcinomatous growths in mice. Whether these compounds have any carcinogenic effect in man is not known.

Ultra-violet light and x-rays are well known carcinogenic agents, yet the controlled application of ultra-violet and the moderate dermatological use of x-ray irradiation are safe and effective therapeutic measures.

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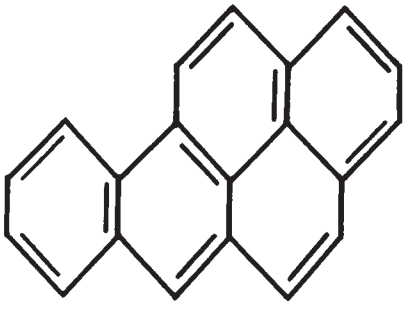
When nearing completion of the present work, we came across the study of Cottini and Mazzone (7), in which these investigators applied a 1% solution of benzpyrene to the normal skin and to various skin lesions of 26 patients daily for 100 days. They noted the following effects: erythema, pigmentation, desquamation, infiltration and verrucous acanthosis, all of which disappeared in a month after stopping the applications. In no case did a cancerous growth appear on the painted areas.

In the present investigation, 3-methylcholanthrene was applied to the normal human epidermis, followed by serial biopsy studies for a period of 7 days. The effect of trauma (croton oil) apparently sensitizes (3, 4, 5) the skin of the mouse to the carcinogenic action of various hydrocarbons (37, 53). Keratin stripped epidermis (Pinkus, 11), was also painted with 3 methylcholanthrene and similarly studied. Cellophane stripping of the stratum corneum produces a well known response and accurate accounts outlining these have been published (11, 12, 40, 49). It was felt that the observation of any modification of this response may serve as a useful means of evaluating the mitotic effects of various compounds including known carcinogens.

This study was undertaken in an attempt to re-evaluate our concepts on carcinogenesis and to study the mitoses stimulating properties of various substances. The firm belief that a single application of any carcinogenic compound to a



BENZO-(*a*) PYRENE

small area of human skin has never been known to produce cancer, and that valuable information may be gained by such work prompted this investigation.

METHOD

Eleven men, five of whom were physicians, between 26 and 51 years of age volunteered for this investigation. All were fully cognizant of the procedure and the investigative problem.

A. The flexor surface of the forearm was chosen as the most convenient site and an area, 1 cm. wide and 4 cm. long, was mapped out with a ball point pencil. The area was painted with a 1% solution of 3-methylcholanthrene in benzene using a camel hair brush. The painted area dried quickly leaving fine yellow dust-like particles on the surface of the skin. Each volunteer was instructed not to wash the area for a period of three days. The painted area was left uncovered. Biopsy specimens were taken with the aid of a 1% xylocaine local anesthetic at varying intervals from 24 hours to 7 days (see table 1).

B. On the same volunteers, an area on the opposite arm was stripped with cellophane tape, using the technic of keratin stripping of Pinkus (11, 12). An area of stripped epidermis, 1 cm. wide and 4 cm. long was outlined with a ball point pencil and immediately painted with a 1% solution of 3-methylcholanthrene in benzene. 2 mm. biopsy specimens were obtained at varying intervals from 1 to 7 days (see table 2). Control studies were performed on two more volunteers using benzene only on the stripped epidermis and biopsies were obtained on the 1st, 2nd, 3rd, and 7th days.

C. A 1% solution of 9,10-dimethyl-1,2-benzanthracene in liquid petrolatum was used on one volunteer, both on keratin stripped skin and on normal epidermis. Biopsy specimens were taken from each area on the 1st, 2nd, 3rd and 7th days. On another area, daily applications were applied for one week. A single application was applied one and two months later and the results observed, both clinically and by serial biopsy studies. This was the only volunteer to receive more than one application of a carcinogenic compound.

TABLE 1

Number of mitoses per field in epidermis painted with 1% solution of 3-methylcholanthrene in benzene

Case No.	Days						
	1	2	3	4	5	6	7
1	.08	.14	.064				.12
2	.006	.02	.035				.18
3	.04	.11	.09				
4	.064	.04	.1				
5	.02	.08	.11	.023	.16	.0083	.006
6	.009	.27	.46				.3
7	.081			0.93			.13
8			.055	.006	.062		
9				.021	.12	.045	
10		.10	.05				.6
11				.54	.26	.13	

Method of counting mitoses

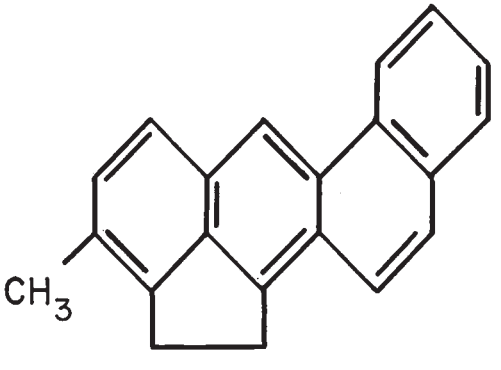
All biopsy specimens were taken with a 1 mm. punch and fixed for 24 hours in Bouin's fluid, dehydrated and embedded in paraffin. Serial sections 8 microns thick were cut and stained with hematoxylin and eosin. The sections examined and the mitoses in the epidermis were counted using an oil immersion objective (890 × magnification) and a square aperture eyepiece. Each field had a diameter of 100 microns and the mitoses in the epidermis were counted in 100 fields. The number of mitoses per field were determined. Judging from the work of Pinkus (11, 12) and previous work on forced epidermal regeneration (40), each field contains approximately 80 to 100 basal and prickle cells. The percentage of mitoses per 100 basal and prickle cells can then be easily calculated. Although this method is open to a rather large margin of error, it was found to give fairly consistent results and is a relatively simple way of appraising the mitotic activity in the epidermis. Strict criteria are of course necessary to give consistent results, the thickness of the sections and the size of the field are of prime importance. In sections not cut vertically and in the presence of marked acanthosis, this method is not applicable.

Only the late prophase through early telephase stages of mitosis were counted. The early prophase is difficult to differentiate from many normal resting nuclei. The final percentage of mitoses per 100 basal and prickle cells was found to be somewhat lower than those obtained by Pinkus (12). He carried out a more elaborate study and measured the epidermal cell nuclei and corrected his final percentage of mitoses by the use of Abercrombie's formula (12).

The figures shown in tables 1 and 2 are the number of mitoses per field. The upper limits of normal were considered to be .04 to .02 mitoses per field. These values are equivalent to 1 mitosis per 25 to 50 fields.

EXPERIMENTAL RESULTS

A. A single application of 3-methylcholanthrene on normal skin did not produce any acute, clinically discernible change. No erythema or increased scaling occurred. The stratum corneum and stratum granulosum appeared unchanged. In most cases an increase in mitoses occurred in the basal and lower prickle cell layer by 48 hours. Increased mitotic activity was present in biopsies taken on the 2nd, 3rd, 4th, 5th, 6th, and 7th days. How long the mitotic unrest persists was not determined.* The present work was limited to the changes occurring during the first 7 days. No consistent pattern in mitotic activity could be found. Marked variations occurred among different individuals. The prickle cell layer increased in thickness by several layers of cells. There was little change in the corium except in most instances an increased perivascular lymphocytic infiltration occurred. Most biopsy specimens showed increased mitotic activity in the upper part of the external root sheath. In several sections, an increased number of mitoses were found in the peripheral cells of the sebaceous glands. Otherwise the sebaceous glands remained unchanged. All biopsy sites healed normally and have been followed for a period of 2 to 8 months without change.



3-METHYLCHOLANTHRENE

* Biopsy specimens taken from one volunteer on the 15th and 21st day after stripping and painting with 1% solution of 3-methylcholanthrene in benzene contained a normal number of mitoses.

TABLE 2

Number of mitoses per field in keratin stripped epidermis painted with 1% solution of 3-methylcholanthrene in benzene

Case No.	Days						
	1	2	3	4	5	6	7
1	0.7	.78	.47				.18
2	.007	.03	1.1				.23
3	.021	1.44	.93	.66	.74	.51	.46
4	.18	.5	1.8				1.08
5	.2			1.8			0.1
6			.039	.006	.14		
7				.3	2.3	.52	
8		2.27	1.1				.67
9				.98	.99	.84	

The upper limits of mitoses found in normal epidermis were considered to be .04 to .02 mitoses per field. This would be equivalent to 1 mitosis per 25 to 50 fields (see text).

B. Applying a 1% solution of 3-methylcholanthrene in benzene to a keratin stripped area of epidermis produced a slight increased erythema in some cases and in several a red flare appeared around the area (49, 40). On gross inspection, no difference could be seen at any time between the results of keratin stripping painted with benzene and the results of keratin stripping, plus the application of 3-methylcholanthrene. Several volunteers developed prolonged slight increase in pigmentation limited to the treated site. However, this may also occur after keratin stripping only.

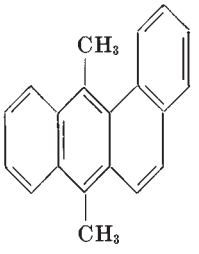
The microscopic appearance after keratin stripping has been fully described by Pinkus (11, 12) and others (40, 49). Keratin stripping and painting with 3-methylcholanthrene produced similar changes except that the mitotic response instead of being greater was slightly lower and prolonged (see table 2). In comparative studies on controls, both in keratin stripped areas and on keratin stripped areas painted with benzene only, the mitotic rise at 48 and 72 hours was found to be higher than after the application of 3-methylcholanthrene. The prickle cell layer increased in width but not appreciably more than after keratin stripping only. There were no striking abnormal nuclear changes (other than those of mitoses) such as were described by Ghosh (8). Except for the occasional mitotic figure in the external root sheath and the

periphery of sebaceous gland cells, these structures showed no change.

In most cases, biopsy material was removed at 1 cm. intervals. However, in 4 volunteers, they were spaced at 2 cm. in order to avoid any overflow of mitotic activity from the trauma of previous biopsies (12, 40).

C. The one volunteer on whom 9,10-dimethyl-1,2-benzanthracene was used both on normal epidermis and on keratin stripped epidermis, produced a mitotic response similar to the studies using 3-methylcholanthrene.

One month later a 1% solution of 9,10-dimethyl-1,2-benzanthracene was applied to a different area and an eczematous reaction ensued. A papulo-vesicular eruption began with marked itching at 48 hours and reached a peak on the 4th day; this began to subside leaving an area of increased pigmentation and slight lichenification still visible by the 8th day. Simultaneously the area treated one month previously (4 inches away) began to itch and fine papulo-vesicles appeared. Biopsy specimens were taken both from the primary and secondary test sites which showed inter-cellular spongiosis and intra-cellular edema. Two months after the initial painting a new test area was chosen and a 1% solution of 9,10-dimethyl-1,2-benzanthracene was applied. A papulo-vesicular reaction occurred in the painted site and also in the area previously painted one month before. The initially treated area of two months previously, became erythematous and itchy but did not develop frank vesicular lesions. Also in this same volunteer allergic sensitivity developed to 1% procaine which produced an immediate wheal on repeated intradermal injection. A patch test to 2% procaine in a water washable ointment base gave a negative result. There was no history of previous allergic sensitivities in this volunteer's personal or family history.



9,10-Dimethyl-1,2-benzanthracene

SUMMARY OF EXPERIMENTAL RESULTS

1. 3-methylcholanthrene painted on the normal intact epidermis caused an increase in mitoses in biopsies taken from 1 to 7 days. No change could be detected on gross examination of the skin.

2. 3-methylcholanthrene painted on areas of keratin stripped epidermis caused a depression in the peak of mitoses compared to the result of stripping only. However, the mitotic count remained high during the period of study, i.e., from 1 to 7 days.

The reaction of keratin stripping, plus painting, with 3-methylcholanthrene produced no appreciable changes clinically, compared to the normal reaction of keratin stripping only. Increased pigmentation was observed in some cases but this also occurs occasionally after ordinary cellophane stripping.

3. An eczematous reaction occurred after multiple applications of 9,10-dimethyl-1,2-benzanthracene in one volunteer. The previously tested sites also became eczematous on subsequent applications to different test areas.

DISCUSSION

Carcinogenesis: general considerations

Tar warts (1) and squamous cell epithelioma occurring in tar workers appear to be dependent of many factors. The length of time of the exposure, as well as the effect of sunlight (1), appear to be important. In cases of tumor-like keratoses on Poth (35), sunlight is at least one of the important etiological factors. Whether the use of tar ointments has ever led to malignant changes in the human being is not known and must be excessively rare in view of the fact that millions of applications are used daily (2).

The possibility exists that kerato-acanthoma (34), tumor-like keratoses of Poth (35) and multiple primary self-healing epithelioma of Ferguson Smith (33) and Acanthomata Appearing After Eczema (52), are all due to the acanthotic action of various agents. Several years ago I saw a patient develop a typical kerato-acanthoma two weeks after superficial x-ray therapy to an area of lichenified seborrhoeic dermatitis on the scalp previously treated with solutions of coal tar. Schaaf and Gross (30) described the acanthosis action in the skin of guinea pigs produced by various ointment bases, including liquid paraffin and soft yellow paraffin. The

response of the human epidermis to carcinogens is quite variable (1) and the results of the present study would also seem to bear out this fact.

A carcinomatous change produces heritable cellular alterations (17). Metabolic pathways that are genetically (17) determined are changed by mutation of nuclear genes. Formation and growth of cancer cells are affected by conditions within the host, the genetic constitution, hormone balance, diet (16, 38) presence of irritation, chemotherapeutic agents, and in fact any factor that affects the environment of the cell.

Berenblum (18) believes that an increase in mitoses may not be the primary cause of a tumorous growth but that delayed maturation allows a sufficient number of undifferentiated daughter cells to accumulate. This hypothesis (18) is not dependent on an increase in growth rate. He states that in some malignant tumors, the mitotic index is low and that the increase in the number of tumor cells is primarily due to delayed maturation.

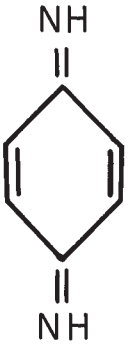
A pre-disposition to develop carcinomatous growths appears to be important as evidenced by the work of Tenchio (27) who produced epidermal neoplastic changes on the normal skin by means of scarification and the application of estrogens in a patient who had multiple basal cell epitheliomas.

A great deal of work has been done on the influence of hormones in regard to their role, both in initiating and enhancing tumorous growths. Sulzberger *et al.* (55) showed that cortisone enhanced the carcinogenic effect of methylcholanthrene in mice and discussed the possible mechanisms whereby cortisone may exert a direct effect on the epidermal cells as well as on the underlying mesenchymal tissues.

The use of macromolecular substances (10), gum acacia, pectin, methyl cellulose, dextran, polyvinyl pyrrolidones, in rats and man result in the development of storage phenomena in different organs. In rats they are associated with tumor-like proliferation of phagocytic cells in the liver, spleen and lymph nodes. It is rather interesting (10) that these "macromolecular" disorders resemble the intra- and extra-cellular storage diseases (xanthomatosis, Gaucher's, Niemann-Pick's Diseases and lipoid proteinosis). In rats and mice these macromolecular substances produce malignant changes in many different body tissues.

Immunological aspects of carcinogenesis

Mayer (20, 21) states that oxidation products of certain allergens and certain carcinogenic agents are the cause of allergic sensitization and in some cases of carcinomatous change (21). The oxidation products (28) formed in the skin combine in the epidermis with nuclear proteins and are fixed in the basal cell. There they act as strong cell stimulants (51). Mayer (21) states that allergic cross-sensitization to compounds such as the aromatic amines (45), nitro derivatives and certain azo dyes (19, 20), sulfonamides (22), PABA and its derivatives and certain polyphenols is due to the metabolic formation of oxidation and reduction products, most likely compounds of quinone structure.



QUINONE DIIMINE

He states (21) that these products represent active allergens and some of these compounds cause atypical epithelial proliferation by the action of the quinone derivatives which have a high affinity for chromosomes. The immunological properties of carcinogens (15) have been demonstrated by antibody precipitation studies in animals. The finding that primary irritants such as croton oil enhance the carcinogenic activity of hydrocarbons actually may be due to the hastened development of allergic sensitivity. However, this is difficult to evaluate, as primary irritants produce malignant growths in mice without the application of carcinogens (41, 44, 46, 47). In the clinical practice of dermatology and in experimental investigations it is a common experience that potential allergens are more effective as sensitizers when in combination with primary irritants.

Sulzberger *et al.* (54) showed that more frequent

applications of methylcholanthrene in lanolin caused tumors to appear later but these were malignant from the beginning with no intermediate precancerous papilloma stage. They (54) also attempted to induce specific sensitization of the skin of guinea pigs to methylcholanthrene and reported weakly positive skin test reactions when challenged 5½ weeks later. In addition to the positive response, erythema and infiltration appeared simultaneously in the previously treated skin areas of four animals.

Jadassohn (13, 29) has shown that sensitizing allergens applied to the guinea pig nipple cause an increased mitotic activity.

Sulfonamides (42) produce sarcomas at the injected site in mice. Sulfonamides and the aniline dye group of carcinogens are chemically related (19). The carcinogenic property of many compounds seems to be more effective if they are also potent allergens.

The study of the mitotic response of many types of compounds may be of value. Hambrick (31) showed that naphthalene derivatives which produce the so called "aniline cancer" in animals (45), stimulated the epithelium of the outer root sheath and the sebaceous gland ducts with marked diminution of the sebaceous gland tissue. In the present study no change occurred in the sebaceous gland. However, several biopsy specimens showed an increased number of mitoses in the peripheral cells of the gland.

Besides animal investigations, studies in man are essential to our better understanding of carcinogenesis. The pitfalls in research have been pointed out by Brues (23). The statistical method of approach and theoretical implications may lead to erroneous conclusions (24, 32). The effects of carcinogenic hydrocarbons vary greatly between animal species (6) and the results of animal experimentation are not entirely applicable to man.

A carcinogenic hydrocarbon, in order to produce a carcinomatous change, must be capable of causing more than just a stimulus to mitosis. The fact that a certain compound acts as a stimulus to the epidermal cells does not necessarily mean it will produce a malignant growth. Physiological processes such as tissue repair, act as powerful mitotic stimulants; the results of which are beneficial to the host.

Apparently many factors are involved to eventuate in a permanent heritable change of a cell and to produce a carcinomatous growth. The work of Bhargava and Heidelberger (50) appears to give conclusive proof that certain carcinogens, or their oxidation products, form protein bound complexes in the epidermal cells. These complexes apparently involve the formation of a quinonoid bond. The immunological status of the host also appears to be intimately associated as a factor playing a role in carcinogenesis.

SUMMARY

1. A 1% solution of 3-methylcholanthrene in benzene was applied to the normal epidermis of 11 volunteers, 5 of whom were physicians. No clinically discernible change occurred. Microscopically, a slight to moderate increase in mitoses was found during the period of study from 1 to 7 days. This mitotic activity showed wide individual variations.

2. A 1% solution of 3-methylcholanthrene in benzene was applied to an area of keratin stripped epidermis. On clinical examination this area did not differ from the results of ordinary cellophane stripping painted with benzene only. Microscopically, the peak of mitoses was depressed but there was a prolonged increase during the period of study from 1 to 7 days.

3. One volunteer received daily applications of 9,10-dimethyl-1,2-benzanthracene in mineral oil for a period of 7 days. One or two months later a single application produced an allergic papulo-vesicular response, both in the test site and the areas previously painted with 9,10-dimethyl-1,2-benzanthracene. This is interpreted as further evidence to support the view that the immunological status of the host is one of the factors involved in carcinogenesis.

4. All cases have been followed for a period of 2 to 8 months. The keratin stripped and painted areas and biopsy sites healed normally.

5. The purpose of this paper is not only to report the effects of carcinogenic hydrocarbons on the human skin but to present a method by which it is possible to study the mitosis stimulating properties of various compounds, many of which are in common use.

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